**ORIGINAL ARTICLE**

**Nicotinamide overload may play a role in the development of type 2 diabetes**

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**METHODS:** Nicotinamide metabolic patterns of 14 diabetic and 14 non-diabetic subjects were compared using HPLC. Cumulative effects of nicotinamide and \(N^2\)-methylnicotinamide on glucose metabolism, plasma \(H_2O_2\) levels and tissue nicotinamide adenine dinucleotide (NAD) contents of adult Sprague-Dawley rats were observed. The role of human sweat glands and rat skin in nicotinamide metabolism was investigated using sauna and burn injury, respectively.

**RESULTS:** Diabetic subjects had significantly higher plasma \(N^2\)-methylnicotinamide levels 5 h after a 100-mg nicotinamide load than the non-diabetic subjects (0.89 ± 0.13 \(\mu\)mol/L vs 0.6 ± 0.13 \(\mu\)mol/L, \(P < 0.001\)). Cumulative doses of nicotinamide (2 g/kg) significantly increased rat plasma \(N^2\)-methylnicotinamide concentrations associated with severe insulin resistance, which was mimicked by \(N^4\)-methylnicotinamide. Moreover, cumulative exposure to \(N^4\)-methylnicotinamide (2 g/kg) markedly reduced rat muscle and liver NAD contents and erythrocyte NAD/NADH ratios.

**CONCLUSION:** These findings suggest that nicotinamide overload, which induced an increase in plasma \(N^4\)-methylnicotinamide, associated with oxidative stress and insulin resistance, plays a role in type 2 diabetes.

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**Key words:** Type 2 diabetes; Nicotinamide; \(N^4\)-methylnicotinamide; Insulin resistance; Oxidative stress; Liver; Sweat glands

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**Abstract**

**AIM:** To investigate whether nicotinamide overload plays a role in type 2 diabetes.

**METHODS:** Nicotinamide metabolic patterns of 14 diabetic and 14 non-diabetic subjects were compared using HPLC. Cumulative effects of nicotinamide and \(N^2\)-methylnicotinamide on glucose metabolism, plasma \(H_2O_2\) levels and tissue nicotinamide adenine dinucleotide (NAD) contents of adult Sprague-Dawley rats were observed. The role of human sweat glands and rat skin in nicotinamide metabolism was investigated using sauna and burn injury, respectively.

**RESULTS:** Diabetic subjects had significantly higher plasma \(N^2\)-methylnicotinamide levels 5 h after a 100-mg nicotinamide load than the non-diabetic subjects (0.89 ± 0.13 \(\mu\)mol/L vs 0.6 ± 0.13 \(\mu\)mol/L, \(P < 0.001\)). Cumulative doses of nicotinamide (2 g/kg) significantly increased rat plasma \(N^2\)-methylnicotinamide concentrations associated with severe insulin resistance, which was mimicked by \(N^4\)-methylnicotinamide. Moreover, cumulative exposure to \(N^4\)-methylnicotinamide (2 g/kg) markedly reduced rat muscle and liver NAD contents and erythrocyte NAD/NADH ratios, and increased plasma \(H_2O_2\) levels. Decrease in NAD/NADH ratio and increase in \(H_2O_2\) generation were also observed in human erythrocytes after exposure to \(N^4\)-methylnicotinamide in vitro. Sweating eliminated excessive nicotinamide (5.3-fold increase in sweat nicotinamide concentration 1 h after a 100-mg nicotinamide load). Skin damage or aldehyde oxidase inhibition with tamoxifen or olanzapine, both being notorious for impairing glucose tolerance, delayed \(N^4\)-methylnicotinamide clearance.

**CONCLUSION:** These findings suggest that nicotinamide overload, which induced an increase in plasma \(N^4\)-methylnicotinamide, associated with oxidative stress and insulin resistance, plays a role in type 2 diabetes.

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INTRODUCTION

Type 2 diabetes, characterized by insulin resistance and oxidative stress\cite{1,2}, has reached epidemic proportions not only among adults, but also among adolescents in the past few decades, which has led to the hypothesis that type 2 diabetes is a result of gene-environment (diet) interactions, because the human genome has not changed markedly in such a short time\cite{3-5}. However, what the environmental/dietary risk factors are and how they function remain unclear.

Nicotinamide, the amide of nicotinic acid, is the precursor for the coenzymes β-nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), which function in many enzyme-catalyzed oxidation and reduction reactions. Therefore, nicotinamide homeostasis is vitally important for the body\cite{6}. In humans, excess nicotinamide is methylated, oxidized or hydroxylated to N\(^1\)-methylnicotinamide, nicotinamide-N-oxide or 6-hydroxynicotinamide, respectively, and then N\(^1\)-methylnicotinamide is further oxidized to the pyridones N\(^1\)-methyl-2-pyridone-5-carboxamide (2Py) and N\(^1\)-methyl-4-pyridone-3-carboxamide by aldehyde oxidase (AOX; EC 1.2.3.1). The major nicotinamide metabolites in human urine are 2Py and N\(^1\)-methylnicotinamide\cite{7}. Although the nicotinamide metabolism pathway is well understood, however, the influence of the catabolic efficiency of excess nicotinamide on the body is not fully understood.

Since pellagra was found to be related to vitamin B\(^1\) (niacin) deficiency in 1930s, more attention is paid to the prevention of niacin deficiency. As a result, nicotinamide is used extensively as a food additive without having undergone full formal safety evaluation\cite{8}, and the chronic effects of nicotinamide overuse are far from understood\cite{9}. Nicotinic acid and nicotinamide frequently are reported to impair glucose tolerance and induce insulin resistance\cite{10-12}. Recent evidence suggests that N\(^1\)-methylnicotinamide is involved in Parkinson’s disease\cite{13}. More importantly, the abrupt increase in prevalence of type 2 diabetes in the United States in the latter half of the 20th century\cite{14} occurred roughly in parallel with the increase in per capita niacin content\cite{15}. A similar situation occurred in China, in which food enrichment with niacin began in 1980s, followed by a sharp increase in the incidence of diabetes from 1% in 1980 to 5.5% in 2001\cite{16}. However, whether type 2 diabetes pathogenesis involves nicotinamide overload is undetermined.

The present study aimed to compare nicotinamide metabolic patterns between diabetic and non-diabetic subjects, and to investigate the relationship between nicotinamide overload and insulin resistance. We found that diabetic subjects exhibited slow N\(^1\)-methylnicotinamide clearance, and that N\(^1\)-methylnicotinamide could trigger oxidative stress and insulin resistance, which suggested that the association of nicotinamide overload with relatively slow N\(^1\)-methylnicotinamide detoxification and excretion might be at least partially responsible for the development of type 2 diabetes.

MATERIALS AND METHODS

Chemicals

Nicotinamide was purchased from Sigma (St. Louis, MO, USA). Nicotinamide tablets (50 mg/tablet) were purchased from Lisheng Pharma (Tianjin, China). N\(^1\)-methylnicotinamide was purchased from Takeda Chemical Industries (Osaka, Japan). Tamoxifen was from Kunshan Sanyou Pharmaceutical Adjuvant Factory (Kunshan, China). Olanzapine was obtained from Beijing Nordhuns Chemical Technology (Beijing, China). N\(^1\)-ethylnicotinamide and 2-Py were synthesized according to the methods described respectively by Hirayama et al\cite{17} and Holman et al\cite{18}.

Nicotinamide load test in humans

This study was approved by the relevant ethics committee, and all the Chinese participants gave informed consent. Fourteen type 2 diabetic patients from eight families with a positive history (eight men and six women, mean age, 55.3 ± 10.2; range, 36-75 years) and 14 age- and sex-matched healthy volunteers without a family history (mean age, 53.8 ± 10.8; range, 39-75 years) participated in this study. Type 2 diabetes was defined as a fasting glucose level ≥ 7.0 mmol/L, or current receipt of hypoglycemic medication. All subjects refrained from drugs, alcohol and caffeinated products for at least 12 h before the study. After an overnight fast, urine was collected and quantitated 1 h before, and 2, 4 and 5 h after loading with 100 mg nicotinamide. Venous blood was collected into sodium citrate tubes before and 5 h after nicotinamide loading, and separated by centrifugation (1500 g, 10 min). Aliquots of each plasma and urine sample were placed directly in liquid nitrogen and then transferred to -80°C and -20°C, respectively.

Cumulative nicotinamide and N\(^1\)-methylnicotinamide experiments

All animal experiments were conducted in accordance with institutional guidelines. Male Sprague-Dawley rats (180-220 g) were fed a standard rat chow. In nicotinamide experiment, rats were divided randomly into three groups (n = 6 each) and fasted for 14 h before the experiment. In the two nicotinamide-treated groups, nicotinamide (100 or 400 mg/kg) was administered (intraperitoneally, ip) and repeated every 2 h for five doses. Glucose tolerance test was performed by injection of glucose (2 g/kg, ip) 2 h after the final nicotinamide injection. Blood glucose was measured 1 h after glucose injection. Blood was collected...
by eye bleed into EDTA tubes under urethane anesthesia (1.5 g/kg, ip) 2 h after glucose administration. Plasma was separated by centrifugation (1500 g, 10 min). After plasma collection, the buffy coat and top fifth reticuloocyte-rich layer of erythrocytes were discarded. Aliquots of plasma and erythrocytes, and harvested samples of liver and gastrocnemius muscle were plunged directly into liquid nitrogen and subsequently stored at -80°C until analysis. The same protocol was used in the N$_1$-methylnicotinamide experiment, except that the two treated groups repeatedly received 100 or 400 mg/kg N$_1$-methylnicotinamide per injection and a total dosage of 0.5 or 2 g, respectively (each group, n = 6).

**AOX inhibition**

Rats in inhibitor-treated groups received subcutaneous tamoxifen (50 mg/kg) or olanzapine (20 mg/kg) twice daily for 4 d, and rats in each control group received vehicle only (each group, n = 6). After an overnight fast, all the rats received nicotinamide (100 mg/kg, ip). Plasma samples were collected 5 h after nicotinamide administration as described above.

**Chronic AOX inhibition**

Rats were divided initially into two groups: tamoxifen-treated (20 mg/kg per day, subcutaneously, n = 24) and vehicle-treated (control, n = 8). Eight control and eight tamoxifen-treated rats were sacrificed at the end of 7 wk. Liver samples were harvested and stored at -80°C for western blotting. The remainder of tamoxifen-treated rats was then divided into two groups treated with tamoxifen (20 mg/kg per day) with or without N$_1$-methylnicotinamide (100 mg/kg per day, subcutaneously), respectively. Two weeks after the treatment, glucose tolerance test was performed by injection of glucose (2 g/kg, ip) after an overnight fast. Blood glucose was measured before (fasting) and 1 h after glucose injection. Samples of plasma, liver and gastrocnemius muscle were harvested under urethane anesthesia (1.5 g/kg, ip) 2 h after glucose injection.

**Thermal injury**

Rats in the burn group (n = 11) were given a 40% total body surface area, full-thickness scald burn under ether anesthesia by immersion of the back in 95°C water for 15 s, as previously described.$^{[18]}$ Sham rats (n = 7) were subjected to an identical procedure, except that they were immersed in 25°C water. All rats received glucose (2 g/kg, ip) 24 h after burning, with an overnight fast. Blood glucose was measured before (fasting) and 1 h after glucose dosing. Samples of plasma, liver and gastrocnemius muscle were harvested 2 h after glucose injection.

**Sweat collection**

Five healthy young male volunteers aged 20-24 years participated in this study. After an overnight fast, whole body sweat was collected before and 1, 2 and 3 h after a single oral dose of 100 mg nicotinamide, by having the volunteers stay in a plastic bag during sauna treatment (80°C, 15 min), with stringent precautions to minimize evaporative loss. Aliquots of sweat were placed in liquid nitrogen, and transferred to -80°C until analysis.

**Assays of glucose, insulin and glycogen**

Blood glucose was measured using a glucometer (OneTouch Ultra; LifeScan Inc.). Plasma insulin was measured by radioimmunoassay using commercial kits (Beijing North Institute of Biological Technology, China). Muscle and liver glycogen contents were determined with Glycogen Assay Kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

**H$_2$O$_2$ assay**

Blood from healthy adult male volunteers was collected in EDTA-containing tubes and centrifuged (1500 g, 10 min). The plasma, buffy coat, and top fifth reticuloocyte-rich layer of erythrocytes were discarded, and the remaining cells were washed three times in isotonic saline, and centrifuged (1500 g, 10 min). After the supernatant was discarded, the packed erythrocytes were transferred to Earle’s Balanced Salt Solution presaturated with 95% O$_2$ and 5% CO$_2$ to make an erythrocyte suspension (20 μL packed erythrocytes/mL). Two hundred microliters of erythrocyte suspension was added to each well of a 96-well plate in the absence or presence of N$_1$-methylnicotinamide or 2Py at concentrations of 10 nmol/L to 100 μmol/L, and incubated for 3 h at 37°C. H$_2$O$_2$ concentrations in the supernatant of cell cultures and in rat plasma were measured using an H$_2$O$_2$ Assay Kit (Beyotime Biotechnology, Jiangsu, China).

**NAD/NADH assay**

Human erythrocyte suspension (20 μL packed erythrocytes/mL Earle’s Balanced Salt Solution) was incubated in 1.5-mL Eppendorf tubes with a 1.5-mm hole in the cover, for 4 h at 37°C in the presence or absence of N$_1$-methylnicotinamide (10 μmol/L). The tubes were centrifuged (1500 g, 15 min, 4°C) and the supernatant was discarded. Four hundred microliters of BioVision NAD/NADH Extraction Buffer (Mountain View, CA, USA) was added to each tube for 5 min to lyse the cells. The lysates were ultrafiltered using BioVision 10-kD cut-off filters (14000 g, 30 min, 4°C). Assays were performed using the NAD$^+$/NADH Quantification Kits according to the manufacturer’s instructions (BioVision). For rat tissue NAD/NADH assay, 20 mg frozen liver, 20 mg frozen muscle or 2 μL packed erythrocytes were homogenized in 400 μL BioVision NAD/NADH Extraction Buffer. The homogenate was ultrafiltered using BioVision 10-kD cut-off filters (14000 g, 30 min, 4°C). The assay was conducted following the manufacturer’s instructions.

**Western blotting**

Western blotting analysis of AOX was performed according to standard protocols. Briefly, 100 μg rat liver proteins were separated on 5%-8% SDS polyacrylamide gels and transferred to polyvinyl difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked in PBS that contained 0.1% Tween-20 and 5% non-fat dry milk for
methylnicotinamide level 5 h after nicotinamide load in the diabetic group (Figure 1B and D). The plasma 5-h total urinary 2Py excretion after 100 mg nicotinamide load in the diabetic group was significantly less than that in the non-diabetic group (20.9 ± 4.5 mg vs. 24.3 ± 4.1 mg, P < 0.05, Figure 1A and C), but urinary N'-methylnicotinamide excretion increased in the diabetic group (Figure 1B and D). The plasma N'-methylnicotinamide level 5 h after nicotinamide load was significantly higher in the diabetic than non-diabetic group (Figure 2). It should be noted that there were no statistical differences in the basal urinary excretions of 2Py and N'-methylnicotinamide and the basal levels of plasma N'-methylnicotinamide between the two groups (Figures 1 and 2). These results suggest that slow plasma N'-methylnicotinamide clearance, which can be revealed by nicotinamide load test, may be a potential biomarker of type 2 diabetes.

Determination of N'-methylnicotinamide, nicotinamide and 2Py
N'-methylnicotinamide, nicotinamide and 2Py were analyzed using an HPLC system that consisted of an LC-9A pump (Shimadzu, Kyoto, Japan), a Rheodyne 7725i sample injector (Rheodyne LLC, Rohnert Park, CA, USA), a Hypersil ODS C18 column (Thermo, Bellefonte, PA, USA), a Waters 470 fluorescence detector (Milford, MA, USA) for N'-methylnicotinamide measurements, and a UV3000 detector (Thermo Separations Products, Fremont, CA, USA) for 2Py detection. N'-methylnicotinamide concentration was analyzed by detecting its fluorescent 1,6-naphthyridine derivatives according to the method of Musfeld et al[9], using 366 nm excitation and 418 nm emission wavelengths, with the mobile phase (10 mmol/L sodium heptanesulfonate, 50 mmol/L triethylamine and 22% acetonitrile in water, pH 3.2 with 85% H3PO4) at a flow rate of 1 mL/min. Nicotinamide was quantitated according to the same procedure after quantitative conversion of nicotinamide to N'-methylnicotinamide using iodomethane, according to the method of Clark[20]. For analyzing urinary 2Py, 50 μL 85% H3PO4 and 0.45 mL water were added to each urine sample (0.5 mL). After brief vortex mixing, the samples were centrifuged (12,000 g, 10 min). The supernatant was filtered through a 0.45-μm filter for HPLC analysis. Standard solutions were prepared for calibration, which contained 0, 0.1, 1, 10 and 100 μg/mL of pure 2Py in normal urine. The mobile phase (5% methanol, 10 mmol/L KH2PO4, 10 mmol/L triethylamine, pH 3.0 adjusted with 85% H3PO4) was delivered at 1 mL/min. UV detection was performed at 260 nm.

Statistical analysis
The data are presented as means ± SD. Statistical differences in the data were evaluated by paired or unpaired Student’s t test, or ANOVA as appropriate, and were considered significant at P < 0.05.

RESULTS

Slow N'-methylnicotinamide clearance is a prominent feature of type 2 diabetes
The 5-h total urinary 2Py excretion after 100 mg nicotinamide load in the diabetic group was significantly less than that in the non-diabetic group (20.9 ± 4.5 mg vs. 24.3 ± 4.1 mg, P < 0.05, Figure 1A and C), but urinary N'-methylnicotinamide excretion increased in the diabetic group (Figure 1B and D). The plasma N'-methylnicotinamide level 5 h after nicotinamide load was significantly higher in the diabetic than non-diabetic group (Figure 2). It should be noted that there were no statistical differences in the basal urinary excretions of 2Py and N'-methylnicotinamide and the basal levels of plasma N'-methylnicotinamide between the two groups (Figures 1 and 2). These results suggest that slow plasma N'-methylnicotinamide clearance, which can be revealed by nicotinamide load test, may be a potential biomarker of type 2 diabetes.

High plasma N'-methylnicotinamide induces insulin resistance
We examined the effect of nicotinamide overload on rat glucose metabolism. Rats treated with cumulative doses of nicotinamide (2 g/kg) exhibited significantly higher levels of blood glucose and plasma insulin, but significantly lower muscle glycogen content than control rats after glucose load (Figure 3A). Another notable change after nicotinamide administration was that there was a marked increase in plasma N'-methylnicotinamide (Figure 3A), so we examined the effects of N'-methylnicotinamide. Cumulative doses of N'-methylnicotinamide (2 g/kg) had comparable effects to nicotinamide (Figure 3B), which suggested that the effects of nicotinamide overload might have been mediated by N'-methylnicotinamide.

N'-methylnicotinamide triggers oxidative stress
Type 2 diabetes is associated with oxidative stress[1,2]. We therefore examined whether nicotinamide overload and high N'-methylnicotinamide levels were implicated in oxidative stress. The results showed that cumulative effects of nicotinamide (2 g/kg) or N'-methylnicotinamide (2 g/kg) led to a significant increase in rat plasma levels of H2O2 (Figure 4A and B), a major reactive oxygen species (ROS) and common indicator of oxidative stress[3]. Such an enhancing effect was also observed in human erythrocytes in vitro at physiological concentrations of N'-methylnicotinamide (Figure 4C), whereas 2Py, the end product of nicotinamide, did not have an enhancing effect at the observed concentrations (10 nmol/L to 100 μmol/L) (data not shown). These results indicate that high plasma N'-methylnicotinamide may induce systemic oxidative stress.

Increasing evidence has indicated that type 2 diabetes has abnormalities in the NAD+/NADH redox couple[21]. We therefore investigated whether N'-methylnicotinamide affected tissue NAD levels. Cumulative exposure to N'-methylnicotinamide significantly reduced rat muscle and liver NAD (NAD+ + NADH) contents (Figure 4D and E). Notably, the erythrocytes of rats treated with cumulative doses of N'-methylnicotinamide (2 g/kg) exhibited a significant increase in NADH and decrease in NAD+/NADH ratio (Figure 4F). A similar effect was observed in human erythrocytes in vitro (Figure 4G). These results suggest that N'-methylnicotinamide-induced oxidative stress may originate from imbalance in the NAD+/NADH redox couple.

AOX inhibition reduces N'-methylnicotinamide clearance
AOX is responsible for conversion of N'-methyli-
cotinamide-to-2Py. We examined changes in N'-methylnicotinamide clearance after rat AOX inhibition by the putative inhibitors tamoxifen and olanzapine, both of which are known to impair glucose tolerance.

The rats treated with tamoxifen (100 mg/kg per day) or olanzapine (40 mg/kg per day) for 4 d exhibited significantly higher plasma N'-methylnicotinamide levels than control rats 5 h after 100 mg/kg nicotinamide
Moreover, chronic tamoxifen treatment for 7 wk significantly reduced rat liver AOX protein expression \( (P < 0.05, \text{Figure } 5C) \). To further explore the role of nicotinamide overload in insulin resistance, we used tamoxifen plus \( N^1 \)-methylnicotinamide to mimic the conditions of relatively low AOX activity and excess nicotinamide intake. Rats treated with tamoxifen plus \( N^1 \)-methylnicotinamide exhibited significantly higher blood glucose and much lower liver glycogen content than those treated with tamoxifen alone after glucose loading (Figure 5D).

**Skin involvement in \( N^1 \)-methylnicotinamide-mediated insulin resistance**

If \( N^1 \)-methylnicotinamide is indeed involved in insulin resistance, then any factors related to \( N^1 \)-methylnicotinamide detoxification and excretion may affect insulin sensitivity. Of the factors, skin may be of particular significance because it takes part in \( N^1 \)-methylnicotinamide detoxification and excretion, respectively, through skin AOX and sweat glands\(^{[35]}\). We thus explored the role of skin by analyzing human sweat excretion of nicotinamide and \( N^1 \)-methylnicotinamide after nicotinamide loading, or by assessing changes in plasma \( N^1 \)-methylnicotinamide clearance of rats with severe skin damage. Importantly, we found that nicotinamide concentrations in the sweat 1 h after 100 mg nicotinamide loading was 5.3-fold higher than that in fasting sweat, whereas \( N^1 \)-methylnicotinamide concentrations were not significantly altered under such conditions (Figure 6A and B). This indicates that excess nicotinamide, without needing any conversion, is expelled through sweat. Moreover, this study found that rats with severe skin damage had a significant elevation in plasma \( N^1 \)-methylnicotinamide associated with high blood glucose and plasma insulin levels, but lower muscle glycogen content after glucose loading (Figure 6C).

**DISCUSSION**

The main findings of this study were that: (1) nicotinamide overload elevates plasma levels of \( N^1 \)-methylnicotinamide associated with oxidative stress and insulin resistance; (2) the skin plays an important role in expelling excess nicotinamide and detoxifying \( N^1 \)-methylnicotinamide; and (3) diabetic subjects have slow plasma \( N^1 \)-methylnicotinamide clearance. These findings may contribute to explain the mechanism of oxidative stress and insulin resistance in a variety of clinical conditions.
Insulin resistance and oxidative stress are the major hallmarks of type 2 diabetes[1,2], but the mechanisms underlying the development of systemic oxidative stress and insulin resistance are unclear. Both nicotinic acid and nicotinamide have been reported to induce insulin resistance, which may lead to elevation of plasma insulin due to β-cell compensation[10-12]. Coincidentally, our data showed that nicotinamide overload induced acute insulin resistance in rats associated with high plasma levels of N1-methylnicotinamide; the methylation product of nicotinamide being more toxic than nicotinamide (>6-fold)[29]. Importantly, diabetic subjects exhibited significantly higher plasma N1-methylnicotinamide levels than non-diabetic subjects after nicotinamide loading, which suggests its involvement in the toxic effects of nicotinamide overload. Indeed, this study demonstrated that N1-methylnicotinamide mimicked the effect of nicotinamide overload, which suggested N1-methylnicotinamide mediation of the toxic effect.

Oxidative stress may induce NAD depletion, a marker of cell injury[34,35]. Indeed, this study found that N1-methylnicotinamide-induced high plasma H2O2 level was associated with a significant reduction in NAD content in muscle and liver (Figure 4). These results suggest that N1-methylnicotinamide is a potent trigger of diabetic oxidative stress.

The oxidative stress induced by nicotinamide overload may be due to NAD depletion, which suggests that N1-methylnicotinamide is a potent trigger of diabetic oxidative stress. The oxidative stress may induce NAD depletion, a marker of cell injury[34,35]. Indeed, this study found that N1-methylnicotinamide-induced high plasma H2O2 level was associated with a significant reduction in NAD content in muscle and liver (Figure 4). These results suggest that N1-methylnicotinamide is a potent trigger of diabetic oxidative stress.

Figure 4 Effects of N1-methylnicotinamide on H2O2 generation and NAD levels. A, B: Cumulative effects of nicotinamide (NM, 0.5 or 2 g/kg) and N1-methylnicotinamide (NMN, 0.5 or 2 g/kg) on rat plasma H2O2 levels; C: H2O2 concentrations in the supernatant of cultured human erythrocytes with or without 3 h exposure to different concentrations of NMN. For each concentration, n = 4; D, E: NAD (NAD+ and NADH) contents in muscle (D) and liver (E) of rats treated with saline (control) or a cumulative dose of 2 g/kg NMN; F: NAD and NADH contents and NAD/NADH ratio in the erythrocytes (RBCs) of rats with or without cumulative exposure to NMN; G: NAD and NADH contents, and NAD/NADH ratio in human RBCs with (n = 4) or without (control, n = 4) 4 h exposure to 10 μmol/L NMN in vitro. *P < 0.05 vs control, **P < 0.01 vs control. Bar graphs indicate mean ± SD.
Figure 5  Effects of aldehyde oxidase (AOX) inhibitors on plasma N\(^1\)-methylnicotinamide levels and glucose metabolism in rats. A and B: Plasma N\(^1\)-methylnicotinamide (NMN) levels 5 h after nicotinamide load (100 mg/kg, ip) in rats treated with or without AOX inhibitors tamoxifen (Tam, A) or olanzapine (Olan, B) (each group, n = 6). \(^b P < 0.05\) vs control, \(^a P < 0.01\) vs control; C: Liver AOX expression in rats with or without 7 wk tamoxifen treatment. The blot is representative of four independent experiments; D: Responses to a glucose tolerance test in rats after 9 wk treatment with tamoxifen with or without NMN (100 mg/kg per day) treatment in the last 2 wk. FBG: Fasting blood glucose; GTT (1 h): Blood glucose measured 1 h after glucose tolerance test. \(^b P < 0.01\) vs control. Bar graph indicates mean ± SD.

Figure 6  Role of skin in nicotinamide metabolism and insulin resistance. A: Representative HPLC chromatograms showing changes of sweat nicotinamide (NM) and N\(^1\)-methylnicotinamide (NMN) concentrations in a subject before and 1, 2 and 3 h after 100 mg nicotinamide loading. 1 and 2 in Aa are NM and internal standard N\(^1\)-ethylnicotinamide, respectively; 1 and 2 in Ab are NMN and internal standard N\(^1\)-ethylnicotinamide, respectively; B: Summary of the measurements shown in A. \(^b P < 0.0001\) vs control; C: Comparison of plasma NMN and insulin levels, muscle and liver glycogen contents, and blood glucose between sham-burn (\(n = 7\)) and burn (\(n = 11\)) rats after glucose load. FBG: Fasting blood glucose; GTT: Blood glucose 1 h after glucose injection. Bar graphs show mean ± SD. \(^c P < 0.05\), \(^d P < 0.01\) vs control.
The muscle and liver of rats. Then came the vital question: where did the excess systemic ROS originate? NADH-dependent ROS generation is an important source of intracellular ROS. Accumulating evidence has indicated that diabetes shows a decreased cytosolic NAD+/NADH ratio in a variety of tissues. Importantly, this study demonstrates that N1-methylnicotinamide decreases NAD+/NADH ratio in rat erythrocytes in vivo and human erythrocytes in vitro. Thus, it is likely that diabetic oxidative stress is initiated by high plasma N1-methylnicotinamide-induced imbalance in the NAD+/NADH redox couple. Many cellular processes are governed by the enzymes using NAD+/NADH as a cofactor, therefore, it is not difficult to understand why a set of metabolic abnormalities happen in type 2 diabetes.

**Role of AOX in insulin resistance**

Mammalian AOX is a molybdo-flavo enzyme involved in the detoxification of various endogenous and exogenous N-heterocyclic compounds. N1-methylnicotinamide is one of the substrates of AOX by which N1-methylnicotinamide is oxidized to pyridones, and thus, detoxified. AOX is expressed predominantly in the liver. Therefore, severe liver disease might be expected to reduce plasma N1-methylnicotinamide clearance and subsequent insulin resistance. In fact, it is well known that liver cirrhosis is associated with high incidence of diabetes. Pumpo et al. have found that cirrhotic patients have high serum N1-methylnicotinamide levels, both in basal values and after nicotinamide loading. Moreover, non-alcoholic steatohepatitis, the most prevalent liver disease in the western world, typically is associated with the metabolic syndrome that is characterized by insulin resistance, diabetes and hypertension. Tamoxifen, a well-known inducer of non-alcoholic steatohepatitis, is found to inhibit strongly AOX activity and its expression (Figure 5C). Collectively, it seems that the decrease in N1-methylnicotinamide detoxification may be involved in hepatogenic insulin resistance.

AOX is also expressed in the skin, which suggests skin involvement in N1-methylnicotinamide detoxification. Moreover, as found in this study, human sweat glands can excrete excess nicotinamide. Therefore, decreased skin function may be implicated in N1-methylnicotinamide-induced insulin resistance. In fact, severe burns may induce long-lasting insulin resistance, a well-documented but poorly understood phenomenon. The present study demonstrated that severe burns significantly delayed N1-methylnicotinamide clearance, which suggests that long-lasting post-burn insulin resistance may involve a decrease in nicotinamide detoxification and excretion.

**Relationship between lifestyle risk factors and nicotinamide overload**

Environmental and lifestyle risk factors may trigger type 2 diabetes. From our study, it emerges that the risk factors may involve nicotinamide overload. Firstly, excess nicotinamide may lead to high plasma N1-methylnicotinamide, with subsequent oxidative stress and insulin resistance. In response to insulin resistance, the pancreatic β cells have to increase insulin secretion (hyperinsulinemia) compensatorily, which may eventually lead to β-cell failure and blood sugar levels being out of control. The result that tamoxifen-induced AOX inhibition plus N1-methylnicotinamide impaired rat glucose tolerance (Figure 5D) implies that excess nicotinamide intake may be more harmful to those with a low N1-methylnicotinamide clearance.

Secondly, dietary risk factors may be related to nicotinamide contents in foods. For example, meat, which is rich in nicotinamide, increases the risk of type 2 diabetes. Moreover, food fortification with niacin may play a role in nicotinamide overload. If comparing the epidemic of type 2 diabetes...
2 diabetes in the United States\[13\] with the history of food fortification\[14\] one can clearly see that the trend for rapid increase in the incidence of diabetes has occurred in parallel with the trend in mandatory niacin-fortification-induced increase in the per capita niacin consumption in the latter half of the 20th century. These facts may explain why the western dietary pattern, characterized by a high intake of meat and niacin-fortified foods, confers such a high risk of type 2 diabetes.

Thirdly, sedentary lifestyle risk factor may involve sweat gland inactivity. Sweat gland activity fluctuates according to ambient temperature; the most significant feature of the gland. Therefore, sweat gland inactivity is expected to slow nicotinamide catabolism and thereby increase the danger of developing insulin resistance. In fact, the cold season is known to worsen glucose metabolism\[3\], whereas exercising sufficiently to sweat may reduce diabetic incidence\[9\]. Therefore, it is likely that sedentary lifestyle risk factors may be at least partially due to sweat gland inactivity by air-conditioned working/living environments.

It should be noted that large doses of nicotinic acid and nicotinamide may induce liver damage\[45,46\], therefore, long-term investigation may be necessary to determine the relationship between chronic nicotinamide overload and non-alcoholic steatohepatitis. Historically, the epidemic of pellagra has been restricted mainly to those who have performed heavy industrial labor with poor nutrient supply\[9\]. Hence, the present study gives rise to an important social and public health issue as to whether foods need to be fortified with niacin (nicotinamide or nicotinic acid), when the people in developed countries have already been living in an age of over-nutrition and sweat gland inactivity. In summary, it appears that gene-environment (diet) interactions may be a reflection, to some extent, of the outcome of combination of nicotinamide overload and relatively low N1-methyl nicotinamide detoxification and excretion. As summarized in Figure 7, the pathogenesis of type 2 diabetes may be at least partially due to long-term excess nicotinamide intake, and/or slowness in N1-methyl nicotinamide detoxification, and/or decrease in excess nicotinamide and N1-methyl nicotinamide excretion. This may lead to high plasma N1-methyl nicotinamide levels, and subsequently oxidative stress and insulin resistance. Therefore, reducing nicotinamide intake and facilitating excretion of nicotinamide metabolites may be a useful preventive and therapeutic intervention in type 2 diabetes.


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